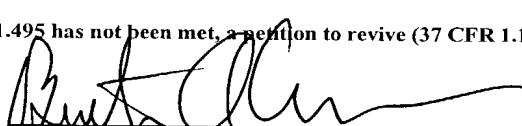


JOINT ROD POT/PTO 24 APR 2001

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		ATTORNEY'S DOCKET NUMBER <u>1581/00265</u> U.S. APPLICATION NO. (Unknown, see 37 CFR 1.15) 09/830111
INTERNATIONAL APPLICATION NO. PCT/JP00/05659	INTERNATIONAL FILING DATE 24 August 2000	PRIORITY DATE CLAIMED 24 August 1999
TITLE OF INVENTION PROCESS FOR PRODUCING COENZYME Q10		
APPLICANT(S) FOR DO/EO/US MATSUDA, Hideyuki, KAWAMUKAI, Makoto, YAJIMA, Kazuyoshi, IKENAKA, Yasuhiro, HASEGAWA, Junzo, TAKAHASHI, Satomi		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none">1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 3712. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1)4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))<ol style="list-style-type: none">a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau)b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))<ol style="list-style-type: none">a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).b. <input type="checkbox"/> have been transmitted by the International Bureau.c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expiredd. <input type="checkbox"/> have not been made and will not be made.8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).10. <input type="checkbox"/> A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).		
Items 11. to 16. below concern other document(s) or information included:		
<ol style="list-style-type: none">11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment14. <input type="checkbox"/> A substitute specification.15. <input type="checkbox"/> A change of power of attorney and/or address letter16. <input checked="" type="checkbox"/> Other items or information. Receipt in the Case of an Original Deposit, ISR, Sequence Listing (paper copy only)		

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO PCT/JP00/05659	ATTORNEY'S DOCKET NUMBER 1581/00265												
<input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTO USE ONLY												
<p>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</p> <table> <tr> <td>Search Report has been prepared by the EPO or JPO.</td> <td>\$860.00</td> </tr> <tr> <td>International preliminary examination fee paid to USPTO (37 CFR 1.482)</td> <td>\$69</td> </tr> <tr> <td>0.00</td> <td></td> </tr> <tr> <td>No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).</td> <td>\$710.00</td> </tr> <tr> <td>Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.</td> <td>\$1,000.00</td> </tr> <tr> <td>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).</td> <td>\$100.00</td> </tr> </table>			Search Report has been prepared by the EPO or JPO.	\$860.00	International preliminary examination fee paid to USPTO (37 CFR 1.482)	\$69	0.00		No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).	\$710.00	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.	\$1,000.00	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).	\$100.00
Search Report has been prepared by the EPO or JPO.	\$860.00													
International preliminary examination fee paid to USPTO (37 CFR 1.482)	\$69													
0.00														
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).	\$710.00													
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.	\$1,000.00													
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).	\$100.00													
ENTER APPROPRIATE BASIC FEE AMOUNT = \$860														
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)) \$														
Claims	Number Filed	Number Extra												
Total Claims	20- 20 =	0	X \$18.00	\$0										
Independent Claims	1- 3 =	0	X \$80.00	\$0										
Multiple dependent claim(s)(if applicable)			+ \$270.00	\$										
TOTAL OF ABOVE CALCULATIONS = \$860														
Reduction by 1/2 for filing by small entity, if applicable.			\$											
SUBTOTAL = \$860														
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)). \$														
TOTAL NATIONAL FEE = \$860														
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +			\$											
TOTAL FEES ENCLOSED = \$860														
			Amount to be: refunded	\$										
			charged	\$										
a. <input checked="" type="checkbox"/> A check in the amount of \$860 to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>22-0185</u> in the amount of \$ _____ to cover the above fees A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Director is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>22-0185</u> . A duplicate copy of this sheet is enclosed.														
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status SEND ALL CORRESPONDENCE TO: Connolly Bove Lodge & Hutz LLP 1990 M Street, N.W., Suite 800 Washington, DC 20036-3425														
 SIGNATURE <u>Burton A. Amernick</u> NAME <u>24,852</u> REGISTRATION NUMBER														

RECEIVED JULY 23 2001

REC'D PCT/PTO 23 JUL 2001

09/830 111 #3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Hideyuki MATSUDA et al.

Serial No.: 09/830,111

Art Unit:

Filed:

Examiner:

For: PROCESS FOR PRODUCING
COENZYME Q10

Atty Docket: 1581/00265

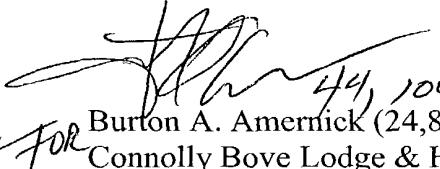
TRANSMITTAL OF CRF OF SEQUENCE LISTING
AND STATEMENT OF IDENTITY

Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to Form PCT/DO/EO/920 mailed in the above-captioned case on May 21, 2001, attached please find a CRF of the Sequence Listing. The contents of the paper copy of the Sequence Listing filed on April 24, 2001 and this CRF of the Sequence Listing are identical and includes no new matter.

Respectfully submitted,


For Burton A. Amernick (24,852)
Connolly Bove Lodge & Hutz LLP
1990 M Street, N.W.
Washington, D.C. 20036-3425
Telephone: 202-331-7111

Date:

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of :
: :
: Hideyuki MATSUDA et al. :
: :
Serial No.: To be assigned : Art Unit: To be assigned
: :
Filed: Herewith : Examiner: To be assigned
: :
For: PROCESS FOR PRODUCING : Atty Docket: 1581/00265
COENZYME Q10 :
: :
: :
: :

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-captioned case as follows.

IN THE CLAIMS

Please amend the claims as follows.

4. (Amended) An expression vector constructed by cloning the DNA according to Claim 1 in an expression vector.
7. (Amended) A transformant as obtainable by transforming a host microorganism with the DNA according to Claim 1.
8. (Amended) A transformant as obtainable by transforming a host microorganism using the expression vector according to Claim 4.

9. (Amended) The transformant according to Claim 7 wherein the host microorganism is *Escherichia coli*.

12. (Amended) A process for producing a coenzyme Q₁₀ which comprises culturing the transformant according to Claim 7 in a culture broth and harvesting the coenzyme Q₁₀ produced and accumulated in the resulting culture.

Please add the following new claims.

13. (New) An expression vector constructed by cloning the DNA according to Claim 3 in an expression vector.

14. (New) A transformant as obtainable by transforming a host microorganism with the DNA according to Claim 3.

15. (New) A transformant as obtainable by transforming a host microorganism using the expression vector according to Claim 5.

16. (New) A transformant as obtainable by transforming a host microorganism using the expression vector according to Claim 6.

17. (New) The transformant according to Claim 8 wherein the host microorganism is *Escherichia coli*.

18. (New) A process for producing a coenzyme Q₁₀ which comprises culturing the transformant according to Claim 8 in a culture broth and harvesting the coenzyme Q₁₀ produced and accumulated in the resulting culture.

19. (New) A process for producing a coenzyme Q₁₀

which comprises culturing the transformant according to Claim 9 in a culture broth

and harvesting the coenzyme Q₁₀ produced and accumulated in the resulting culture.

20. (New) A process for producing a coenzyme Q₁₀

which comprises culturing the transformant according to Claim 10 in a culture broth

and harvesting the coenzyme Q₁₀ produced and accumulated in the resulting culture.

R E M A R K S

The claims have been amended to eliminate multiple dependency and to improve their format. None of these amendments is believed to involve any new matter. Accordingly, it is respectfully requested that the foregoing amendments be entered, that the application as so amended receive an examination on the merits, and that the claims as now presented receive an early allowance.

Respectfully submitted,



Burton A. Amernick (24,852)
Connolly Bove Lodge & Hutz LLP
1990 M Street, N.W., Suite 800
Washington, D.C. 20036-3425
Telephone: 202-331-7111

Date: 4-24-01

APPENDIX – MARKED UP VERSION

4. (Amended) An expression vector constructed by cloning the DNA according to Claim 1 [or 3] in an expression vector.
7. (Amended) A transformant as obtainable by transforming a host microorganism with the DNA according to Claim 1 [or 3].
8. (Amended) A transformant as obtainable by transforming a host microorganism using the expression vector according to Claim 4[, 5 or 6].
9. (Amended) The transformant according to Claim 7 [or 8] wherein the host microorganism is *Escherichia coli*.
12. (Amended) A process for producing a coenzyme Q₁₀ which comprises culturing the transformant according to Claim 7[, 8, 9, 10 or 11] in a culture broth and harvesting the coenzyme Q₁₀ produced and accumulated in the resulting culture.

JC20 Rec'd PCT/PTO 19 APR 2002



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: : Conf. No.: 3002

: Hideyuki MATSUDA et al.

: Serial No.: 09/830,111

: Int'l FD: August 24, 2000

For: PROCESS FOR PRODUCING : Atty Docket: 21581/0265
COENZYME Q10**RESPONSE AND AMENDMENT**

Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notification of Missing Requirements dated February 19, 2002 (copy attached), attached are substitute paper and CRF copies of the sequence listing. These do not introduce any new matter. The contents of these paper and CRF copies of the sequence listing are identical.

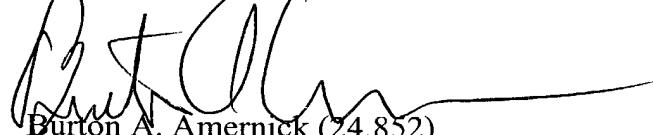
Please enter the paper copy of the sequence listing into the specification.

In view of the above, consideration and allowance are, therefore, respectfully solicited.

In the event the Examiner believes an interview might serve to advance the prosecution of this application in any way, the undersigned attorney is available at the telephone number noted below.

The Director is hereby authorized to charge any fees, or credit any overpayment, associated with this communication, including any extension fees, to CBLH Deposit Account No. 22-0185.

Respectfully submitted,



Burton A. Amernick (24,852)
Customer Number 30678
Connolly Bove Lodge & Hutz LLP
1990 M Street, N.W., Suite 800
Washington, D.C. 20036-3425
Telephone: 202-331-7111

Date: 4-19-02

Sequence listing

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PCT09

RAW SEQUENCE LISTING
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TIME: 16:01:21

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3 <110> APPLICANT: Kaneka Corporation
 5 <120> TITLE OF INVENTION: Process for producing coenzyme Q10
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 9 <150> PRIOR APPLICATION NUMBER: JP P1999-237561
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 15 <211> LENGTH: 1653
 16 <212> TYPE: DNA
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 24 aaatccatat acc atg gcc tca cca gca ctg cgg ata cga agc atc agc 169
 25 Met Ala Ser Pro Ala Leu Arg Ile Arg Ser Ile Ser
 26 1 5 10
 28 tct cga tca atc gcc tct ctg cga tcg gtt acc cta aga aca gcc tcg 217
 29 Ser Arg Ser Ile Ala Ser Leu Arg Ser Val Thr Leu Arg Thr Ala Ser
 30 15 20 25
 32 gca cct tca tta cga cta aga tgt acc ccg acg agc cgg cca tcg agt 265
 33 Ala Pro Ser Leu Arg Leu Arg Cys Thr Pro Thr Ser Arg Pro Ser Ser
 34 30 35 40
 36 tca tgg gct gct gtg tct tcg gcg tcg aga ctg gtt gag cct gat 313
 37 Ser Trp Ala Ala Val Ser Ser Ala Ser Arg Leu Val Glu Pro Asp
 38 45 50 55 60
 40 ccg aat caa cct ctc atc aat ccg ctc aac ttg gtc ggt ccc gag atg 361
 41 Pro Asn Gln Pro Leu Ile Asn Pro Leu Asn Leu Val Gly Pro Glu Met
 42 65 70 75
 44 tca aat ctt aca tcc aac atc cga tct ctc ctc ggt tca gga cac cct 409
 45 Ser Asn Leu Thr Ser Asn Ile Arg Ser Leu Leu Gly Ser Gly His Pro
 46 80 85 90
 48 tct ctc gac act gtc gct aaa tac tat gtt cag tct gag gga aag cat 457
 49 Ser Leu Asp Thr Val Ala Lys Tyr Tyr Val Gln Ser Glu Gly Lys His
 50 95 100 105
 52 att cgt ccg ctc atg gta ctg ctg atg gct cag gcg acg gag gtt gcg 505
 53 Ile Arg Pro Leu Met Val Leu Leu Met Ala Gln Ala Thr Glu Val Ala
 54 110 115 120
 56 cca aaa gtt cag ggt tgg gag aag gtc gtg gag gtt ccg gtg aac gag 553
 57 Pro Lys Val Gln Gly Trp Glu Lys Val Val Glu Val Pro Val Asn Glu
 58 125 130 135 140
 60 gga ctc gca cca cca gag gtg ctc aat gac aag aac cca gat atg 601

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DATE: 06/04/2002

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65	Asn	Met	Arg	Ser	Gly	Pro	Leu	Thr	Lys	Asp	Gly	Glu	Ile	Glu	Gly	Gln		
66								160		165						170		
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70								175		180						185		
72	atg	atc	cat	gca	gca	tca	ctc	ctc	cac	gac	gac	gtt	atc	gac	gct	tcc	745	
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76	gag	acc	aga	cga	aac	gca	cca	tcc	gga	aac	cag	gca	ttc	gga	aac	aag	793	
77	Glu	Thr	Arg	Arg	Asn	Ala	Pro	Ser	Gly	Asn	Gln	Ala	Phe	Gly	Asn	Lys		
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93	Asp	Asp	Ala	Ile	Glu	Ala	Thr	Ala	Thr	Gln	Glu	Thr	Phe	Asp	Tyr	Tyr		
94								270		275						280		
96	ttg	cag	aag	act	tac	ttg	aag	act	gcg	tcc	ttg	att	gcc	aag	tcg	tgc	1033	
97	Leu	Gln	Lys	Thr	Tyr	Leu	Lys	Thr	Ala	Ser	Leu	Ile	Ala	Lys	Ser	Cys		
98								285		290			295			300		
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101	Arg	Ala	Ser	Ala	Leu	Leu	Gly	Gly	Ala	Thr	Pro	Glu	Val	Ala	Asp	Ala		
102								305		310						315		
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105	Ala	Tyr	Ala	Tyr	Gly	Arg	Asn	Leu	Gly	Leu	Ala	Phe	Gln	Ile	Val	Asp		
106								320		325						330		
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110								335		340						345		
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117	Trp	Lys	His	His	Ala	Glu	Leu	Gly	Pro	Met	Ile	Lys	Arg	Lys	Phe	Ser		
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121	Asp	Pro	Gly	Asp	Val	Glu	Arg	Ala	Arg	Glu	Leu	Val	Glu	Lys	Ser	Asp		
122								385		390						395		
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133	Glu Gln Leu Thr Asp Lys Val Leu Thr Arg Ser Arg		
134	430	435	440
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154	20 25 30		
155	Ser Leu Arg Leu Arg Cys Thr Pro Thr Ser Arg Pro Ser Ser Ser		
156	35 40 45		
157	Trp Ala Ala Ala Val Ser Ser Ala Ser Arg Leu Val Glu Pro Asp		
158	50 55 60		
159	Pro Asn Gln Pro Leu Ile Asn Pro Leu Asn Leu Val Gly Pro Glu		
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164	95 100 105		
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188	275	280	285
189	Gln Lys Thr Tyr Leu Lys Thr Ala Ser	Leu Ile Ala Lys Ser	Cys
190	290	295	300
191	Arg Ala Ser Ala Leu Leu Gly Gly Ala	Thr Pro Glu Val Ala	Asp
192	305	310	315
193	Ala Ala Tyr Ala Tyr Gly Arg Asn Leu	Gly Leu Ala Phe Gln	Ile
194	320	325	330
195	Val Asp Asp Met Leu Asp Tyr Thr Val	Ser Ala Thr Asp Leu	Gly
196	335	340	345
197	Lys Pro Ala Gly Ala Asp Leu Gln Leu	Gly Leu Ala Thr Ala	Pro
198	350	355	360
199	Ala Leu Phe Ala Trp Lys His His Ala	Glu Leu Gly Pro Met	Ile
200	365	370	375
201	Lys Arg Lys Phe Ser Asp Pro Gly Asp	Val Glu Arg Ala Arg	Glu
202	380	385	390
203	Leu Val Glu Lys Ser Asp Gly Leu Glu	Lys Thr Arg Ala Leu	Ala
204	395	400	405
205	Glu Glu Tyr Ala Gln Lys Ala Leu Asp	Ala Ile Arg Thr Phe	Pro
206	410	415	420
207	Glu Ser Pro Ala Arg Lys Ala Leu Glu	Gln Leu Thr Asp Lys	Val
208	425	430	435
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VERIFICATION SUMMARY

PATENT APPLICATION: **US/09/830,111A**

DATE: 06/04/2002

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L:9 M:270 C: Current Application Number differs, Replaced Current Application No
L:9 M:271 C: Current Filing Date differs, Replaced Current Filing Date

31PRTS

097830111
JC18 Rec'd PCT/PTO 24 APR 2001

1

SPECIFICATION

PROCESS FOR PRODUCING COENZYME Q₁₀

5

TECHNICAL FIELD

The present invention relates to a process for producing a coenzyme Q₁₀ for pharmaceutical and other uses. More particularly, the invention relates to a process for producing coenzyme Q₁₀ which comprises isolating a gene coding for the 10 coenzyme Q₁₀ side-chain synthase, which is a key enzyme involved in the biosynthesis of coenzyme Q₁₀, i.e. decaprenyl diphosphate synthase, from a fungal strain of the genus Saitoella and introducing it into a host microorganism to let it elaborate coenzyme Q₁₀.

15

BACKGROUND ART

The conventional technology for commercial production of coenzyme Q₁₀ comprises isolating the coenzyme from a tobacco or other plant and modifying the length of its side chain by 20 a synthetic technique.

While it is known that coenzyme Q₁₀ is produced by a broad spectrum of organisms ranging from microorganisms, such as bacteria and yeasts, to higher animals and plants, the method comprising culturing a microorganism and extracting coenzyme 25 Q₁₀ from the microorganism is regarded as one of the most effective production methods and has actually been exploited commercially. However, the prior art methods are invariably poor in productivity, providing for only low outputs and/or involving time-consuming procedures.

30

The pathways for biosynthesis of coenzyme Q₁₀ in organisms are partly different between the prokaryote and the eukaryote but invariably comprise a complicated cascade of reactions involving many kinds of enzymes. However, these pathways are basically comprised of three fundamental steps, namely the step 35 of synthesizing decaprenyl diphosphate as the precursor of the

prenyl side-chain of coenzyme Q₁₀, the step of synthesizing p-hydroxybenzoic acid as the basis of the quinone ring of coenzyme Q₁₀, and the step of coupling these two compounds together and effecting a serial substituent transformation to 5 complete coenzyme Q₁₀. Of these reactions, the reaction determinant of the length of the side-chain of coenzyme Q₁₀ and acknowledged to be the rate-determining step of its biosynthesis, i.e. the reaction catalyzed by decaprenyl diphosphate synthase, is considered to be the most important 10 reaction. Therefore, in order that coenzyme Q₁₀ may be produced with good efficiency, it seems worthwhile to isolate the key gene involved in said biosynthesis, namely the gene coding for decaprenyl diphosphate synthase, and utilize it for enhanced 15 production of the enzyme. As sources of the gene, fungi capable of producing coenzyme Q₁₀ in comparatively large amounts can be regarded as useful candidates.

Heretofore, genes coding for decaprenyl diphosphate synthase have been isolated from several kinds of microorganisms, such as Schizosaccharomyces pombe (JP09-173076A) and Gluconobacter suboxydans (JP10-57072A), etc., but the inherent coenzyme Q₁₀ productivity of these microorganisms cannot be considered high enough and neither an efficient cultural protocol for these microorganisms nor an efficient isolation and purification procedure has been established as yet. Therefore, there has been a standing demand for isolation of a coenzyme Q₁₀-encoding gene from a microorganism capable of highly producing a coenzyme Q₁₀.

30 Devoted to providing a solution to the above-mentioned production problems, the present invention has for its object to isolate a gene coding for the enzyme synthesizing the coenzyme Q₁₀ side chain from a fungal strain of the genus Saitoella and exploit it to advantage for the efficient microbial production of coenzyme Q₁₀.

To accomplish the above object, in the present invention, the key gene involved in the biosynthesis of coenzyme Q₁₀, namely the gene coding for decaprenyl diphosphate synthase, was isolated from a fungal strain of the genus Saitoella in the first 5 place. Then, this gene was introduced and allowed to be expressed in a host microorganism, such as Escherichia coli, to thereby enable the host to produce coenzyme Q₁₀ with efficiency.

The inventors of the present invention made intensive 10 investigations for isolating such genes coding for decaprenyl diphosphate synthase from fungal strains of the genus Saitoella capable of producing comparatively large amounts of coenzyme Q₁₀ and have succeeded in isolating said genes.

The present invention, therefore, is concerned with a DNA 15 of the following (a), (b) or (c).

(a) a DNA having the nucleotide sequence shown under SEQ ID NO:1

(b) a DNA having a nucleotide sequence derived from the nucleotide sequence of SEQ ID NO:1 by the deletion, addition, 20 insertion and/or substitution of one or a plurality of nucleotides

and coding for a protein having decaprenyl diphosphate synthase activity

(c) a DNA which hybridizes with the DNA having the nucleotide 25 sequence of SEQ ID NO:1 under a stringent condition

and codes for a protein having decaprenyl diphosphate synthase activity.

The present invention is further concerned with a protein of the following (d) or (e).

30 (d) a protein having the amino acid sequence shown under SEQ ID NO:2

(e) a protein having an amino acid sequence derived from the amino acid sequence of SEQ ID NO:2 by the deletion, addition, insertion and/or substitution of one or a plurality of amino 35 acids

and having decaprenyl diphosphate synthase activity.

The invention is further concerned with a DNA coding for this protein.

The present invention is further concerned with an expression vector containing said DNA. For the expression vector of the invention, various vector systems heretofore known can be utilized and, therefore, may for example be pNTS1 as constructed by cloning the DNA having the sequence of SEQ ID NO:1 into the vector pUCNT for expression.

10 The present invention is further concerned with a transformant as constructed by transforming a host microorganism with said DNA. As the host microorganism for the invention, Escherichia coli can be used with advantage.

The invention is further concerned with a process for
15 producing coenzyme Q₁₀

which comprises culturing said transformant in a culture broth and harvesting the coenzyme Q_{10} produced and accumulated in the resulting culture. The host microorganism for this process is not particularly restricted but may be Escherichia coli to mention a preferred example. The coenzyme Q produced by Escherichia coli is coenzyme Q₈ but the invention enables this microorganism to produce coenzyme Q₁₀.

The inventors made intensive investigations on the isolation of the enzyme gene from a fungal strain which belongs to the genus Saitoella and is capable of producing comparatively large amounts of coenzyme Q₁₀ and succeeded in acquiring a fragment of the particular gene by a PCR technique.

The inventors compared the sequence of the known gene coding for decaprenyl diphosphate synthase with the genes 30 coding for polyprenyl diphosphate synthases, namely long-chain prenyl synthases which are analogous to said known enzyme gene but differ from the same in chain length and, for the region of high homology, synthesized various PCR primers. Using these primers in various combinations, they studied PCR conditions. 35 As a result, they found by analysis of the gene sequence that

when a PCR using DPS-1 (5'-AAGGATCCTNYTNCAYGAYGAYGT-3') and DPS-1 1AS (5'-ARYTGNADRAAYTCNCC-3') [in the above sequences, R means A or G; Y means C or T, and N means G, A, T or C] as primers is carried out according to the protocol of heat-
5 treatment at 94 °C × 3 minutes, followed by 40 cycles of 94 °C, 1 minute → 43°C, 2 minutes → 72°C, 2 minutes, a ca 220 bp fragment of the enzyme gene can be amplified from the chromosome gene of Saitoella complicata IFO 10748, a fungus belonging to the genus Saitoella.

10 Then, to acquire the full length of this enzyme gene, the chromosome gene of Saitoella complicata IFO 10748 is digested with the restriction enzyme EcoRI and inserted into a λ phage vector to construct a recombinant phage library. After the plaque is transferred to a nylon membrane, the plaque
15 hybridization is carried out using the labeled PCR fragment, whereby a clone having the full-length decaprenyl diphosphate synthase gene can be obtained.

20 Sequencing of the decaprenyl diphosphate synthase gene occurring in the above clone reveals that the gene has the nucleotide sequence shown under SEQ ID NO:1 of SEQUENCE LISTING. The amino acid sequence deduced from the above nucleotide sequence is shown under SEQ ID NO:2. Here, a sequence characteristic of a gene coding for decaprenyl diphosphate synthase is observed.

25 The DNA of the invention may be any of the DNA having the nucleotide sequence shown under SEQ ID NO:1, the DNA having a nucleotide sequence derived from the sequence of SEQ ID NO:1 by the deletion, addition, insertion and/or substitution of one or a plurality of nucleotides and coding for a protein having 30 decaprenyl diphosphate synthase activity, and the DNA which hybridizes with the DNA having the nucleotide sequence of SEQ ID NO:1 under a stringent condition and codes for a protein having decaprenyl diphosphate synthase activity.

35 The "nucleotide sequence derived by the deletion, addition, insertion and/or substitution of one or a plurality

of nucleotides" means any nucleotide sequence derived by the deletion, addition, insertion and/or substitution of a number of nucleotides of the order which can be deleted, added, inserted and/or substituted by the methods well known in the art, for example as described in, inter alia, Protein, Nucleic Acid, Enzyme, Supplemental Issue: Gene Amplification PCR Technology TAKKAI 35 (17), 2951-3178 (1990) and Henry A. Erlich (ed.), PCR Technology (the translation edited by Ikunoshin Kato) (1990).

10 As used in this specification, the term "protein having decaprenyl diphosphate synthase activity" means a protein capable of synthesizing decaprenyl diphosphate in a yield of not less than 10%, preferably not less than 40%, more preferably not less than 60%, still more preferably not less than 80%,
15 relative to the protein having the amino acid sequence shown under SEQ ID NO:2. Such yield measurements can be made by the technique which comprises reacting FDP (farnesyl diphosphate) and ¹⁴C-IPP (radiolabeled isopentenyl diphosphate) with the enzyme of interest, hydrolyzing the resulting ¹⁴C-DPP
20 (decaprenyl diphosphate) with phosphatase, fractionating the hydrolysate by TLC, and assaying the amounts taken up in spots corresponding to the respective chain lengths (Okada et al., Eur. J. Biochem., 255, 55 to 59).

25 The "DNA which hybridizes with the DNA having the nucleotide sequence of SEQ ID NO:1 under a stringent condition" means a DNA obtained by colony hybridization, plaque hybridization, Southern hybridization or the like hybridization technique using the DNA having the nucleotide sequence of SEQ ID NO:1 as the probe. Anyone skilled in the 30 art may easily acquire the objective DNA by carrying out said hybridization according to the methods described in Molecular Cloning, 2nd Edition (Cold Spring Harbor Laboratory Press, 1989).

The protein of the present invention may have the amino acid sequence shown under SEQ ID NO:2 or an amino acid sequence

derived from the amino acid sequence shown under SEQ ID NO:2 by the deletion, addition, insertion and/or substitution of one or a plurality of amino acids and having decaprenyl diphosphate synthase activity.

5 "The amino acid sequence derived by the deletion, addition, insertion and/or substitution of one or a plurality of amino acids" can be obtained by effecting such deletion, addition, insertion and/or substitution by the technology well known in the art, such as a region-specific mutagenesis
10 technique. Specific procedures are described in Nucleic Acid Res. 10, 6487 (1982), Methods in Enzymology, 100, 448 (1983) and other literature.

15 The protein of the present invention preferably has an amino acid sequence showing a homology of not less than 60%, preferably not less than 70%, more preferably not less than 80%, still more preferably not less than 90%, further still more preferably not less than 95%, to the amino acid sequence shown under SEQ ID NO:2.

20 The "homology" is calculated by aligning two nucleotide sequences to be compared in the optimum format, counting the matched base positions (A, T, C, G, U or I) between the two sequences, dividing the count by the total number of bases compared, and multiplying the product by 100. Specifically, this calculation can be made using an analytical software such
25 as Hitachi Soft Engineering's DNASIS, Software Development's GENETYX, or Finland CSC's Clustal X, for instance.

20 While the gene coding for decaprenyl diphosphate synthase must be ligated downstream of a suitable promoter for expression, an expression vector can be constructed, for example by excising
30 a DNA fragment containing the gene with a restriction enzyme or amplifying the enzyme-encoding gene selectively by PCR, followed by cloning it into a vector having a promoter. In the present invention, the expression vector into which the DNA coding for the protein having decaprenyl diphosphate synthase
35 activity may be inserted is not particularly restricted but may

for example be one constructed by ligating a suitable promoter to a plasmid derived from E. coli. The plasmid of E. coli origin includes pBR322, pBR325, pUC19 and pUC119, while the promoter includes T7 promoter, trp promoter, tac promoter, lac promoter 5 and λ PL promoter. Further, as the expression vector of this invention, pGEX-2T, pGEX-3T, pGEX-3X (all from Pharmacia), pBluescript, pUC19 (from Toyobo), pMALC2, pET-3T and pUCNT (described in WO 94/03613), etc. can also be mentioned. Among these, pUCNT can be used with advantage. To mention a specific 10 example, the vector pNTSal for the expression of a decaprenyl diphosphate synthase gene can be constructed by inserting the gene having the DNA sequence shown under SEQ ID NO:1 into the expression vector pUCNT.

Then, this enzyme gene expression vector is introduced 15 into a suitable microorganism, whereby the microorganism is rendered capable of producing coenzyme Q_{10} . The host microorganism is not particularly restricted but Escherichia coli can be used with advantage. The Escherichia coli is not particularly restricted but includes such strains as XL1-Blue, 20 BL-21, JM109, NM522, DH5 α , HB101 and DH5, among others. Among these, E. coli DH5 α can be used with particular advantage. For example, when the expression vector pNTSal containing the 25 decaprenyl diphosphate synthase gene is introduced into this E. coli strain, the coenzyme Q_{10} , which the intact E. coli inherently does not produce, can be produced in a large amount. This E. coli DH5 α (pNTSal) has been deposited with National Institute of Bioscience and Human-Technology (Higashi 1-1-3, Tsukuba-shi, Ibaraki, Japan) under the accession number of FERM BP-6844.

30 Furthermore, Escherichia coli K0229 (Journal of Bacteriology, 179, 3058-3060 (1997), the octaprenyl diphosphate synthase gene-knockout E. coli strain constructed by Kawamukai et al. as the host microorganism, is incapable of producing coenzyme Q_8 and can be utilized as the host for higher 35 production of coenzyme Q_{10} .

The gene can be used not only singly but may be introduced together with another biosynthesis-related gene into a microorganism to thereby obtain still more satisfactory results.

5 Coenzyme Q₁₀ can be produced by culturing the transformant obtained according to the invention and harvesting the product coenzyme Q₁₀ in a per se known manner. When the host microorganism is a strain of Escherichia coli, either LB broth or M9 broth containing glucose and casamino acids can be used
10 as the culture broth. In order that the promoter may be allowed to function with efficiency, the broth may be supplemented with a certain chemical such as isopropyl-thiogalactoside or indolyl-3-acrylic acid. Culture can be carried out at 37 °C for 17 to 24 hours, for instance, optionally under aeration or
15 agitation. In the practice of the invention, the product coenzyme Q₁₀ may be used after purification or as it is in the crude form, depending on the intended use. Isolation of coenzyme Q₁₀ from the culture can be made by using known separation and purification procedures in a suitable
20 combination. As such known separation and purification procedures, there can be mentioned techniques utilizing solubilities, such as salting-out and solvent precipitation; techniques chiefly utilizing differences in molecular weight, such as dialysis, ultrafiltration, gel filtration and
25 (SDS-)polyacrylamide gel electrophoresis; techniques utilizing differences in charge, such as ion exchange chromatography; techniques utilizing specific affinity, such as affinity chromatography; techniques utilizing differences in hydrophobicity, such as reversed-phase high performance
30 liquid chromatography; and techniques utilizing differences in isoelectric point, such as isoelectric focusing, among others.

The use for the coenzyme Q₁₀ obtained according to the invention is not particularly restricted but the enzyme can be applied to pharmaceuticals with advantage.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a restriction map of the pNTSal vector containing the decaprenyl diphosphate synthase gene.

Fig. 2 is an HPLC chart of the coenzyme Q₁₀ produced by 5 the recombinant Escherichia coli DH5 α as transformed with the decaprenyl diphosphate synthase gene.

Fig. 3 is an HPLC chart of the coenzyme Q₁₀ produced by the recombinant Escherichia coli K0229 as transformed with the decaprenyl diphosphate synthase gene.

10

BEST MODE FOR CARRYING OUT THE INVENTION
(Example 1)

The chromosome DNA of Saitoella complicata IFO 10748 was prepared by the method of C. S. Hoffman et al. (Gene, 57 (1987), 15 267-292). Based on the homology to the known long-chain prenyl diphosphate synthase genes, PCR primers, i.e. DPS-1 (5'-AAGGATCCTNYTNCAYGAYGAYGT-3') and DPS-1 1AS (5'-ARYTGNADRAAYTCNCC-3'), were designed. In the above sequences, R stands for A or G; Y for C or T; and N for G, A, T or C. Using 20 these primers, PCR was carried out under the conditions of heat treatment at 94 °C, 3 min. followed by 40 cycles of 94 °C, 1 min. → 43 °C, 2 min. → 72 °C, 2 min., and the PCR product was analyzed by 1.2% agarose gel electrophoresis.

The ca 220 bp fragment thus obtained was excised from the 25 gel and purified using a DNA extraction kit (Sephaglas™ BrandPrep Kit, Amersham Pharmacia Biotech). Then, using a PCR product direct cloning kit (pT7BlueT-Vector Kit, NOVAGEN), the DNA was cloned into the E. coli expression vector to give pT7-SaDPS. Then, using a DNA sequencer (Model 377, Perkin-30 Elmer) and a DNA sequencing kit (Perkin-Elmer; ABI PRISM™ BigDye™ Terminator Cycle Sequence Ready Reaction Kit with AmptiTaq™ DNA Polymerase, FS), DNA sequencing was carried out according to the kit manufacturer's protocol. As a result, there was obtained a sequence corresponding to the nucleotides 35 717 through 924 of SEQ ID NO:1 under SEQUENCE LISTING. The

translation sequence thus obtained contained "GDFLLGRA" which is a characteristic region of polyprenyl diphosphate synthases and, therefore, was considered to be part of the decaprenyl diphosphate synthase gene.

5

(Example 2)

Using 0.03 μ g of a pT7-SaDPS vector containing a 220 bp DNA fragment which was considered to be the decaprenyl diphosphate synthase gene of Saitoella complicata IFO 10748, 10 PCR using primers Sa-1S (which has the sequence of 5'-GAGACCAGACGAAACGCACCA-3') and Sa-2AS (which has the sequence of 5'-TGGTGCCTTCGTCTGGTCTC-3') was carried out [94 °C, 3 min. → (94 °C, 30 sec. → 55 °C, 30 sec. → 72 °C, 1 min.) × 25 cycles → 72 °C, 5 min. → 4 °C]. The PCR product was subjected to gel 15 electrophoresis using 1.2% agarose (Takara) and a ca 145 bp fragment was excised from the gel and purified using a DNA extraction kit (Sephaglas™ BrandPrep Kit; Amersham Pharmacia Biotech). Using about 100 ng of this DNA fragment, 20 chemiluminescence labeling was performed using ECL Direct Nucleic Acid Labeling System (Amersham Pharmacia Biotech).

(Example 3)

The chromosome DNA of Saitoella complicata IFO 10748 was digested with the restriction enzyme EcoRI and electrophoresed 25 through 0.8% agarose gel. This gel was denatured with alkali (0.5 M NaOH, 1.5 M NaCl) and neutralized (0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl), after which HYBOND N + filter (Amersham) was placed on the gel and the Southern transfer was carried out using 20 × SSC overnight. This filter was dried and heated at 80 °C 30 for 2 hours and using ECL Direct Nucleic Acid Labeling/Detection System (Amersham Pharmacia Biotech), Southern hybridization and detection were carried out. Thus, using Gold Hybridizatioon Solution (Amercham Pharmacia Biotech), prehybridization was performed at 42 °C for 1 hour.

35

The chemiluminescence-labeled probe was heated at 95 °C

for 5 minutes, quenched on ice, and added to the prehybridization solution used for filter prehybridized and the hybridization was carried out at 42 °C for 22 hours. This filter was washed with 0.5 × SSC solution containing 6 M urea and 0.4% 5 SDS at 42 °C twice for 20 minutes each and, then, washed with 2 × SSC solution at room temperature twice for 5 minutes each. This filter was immersed in Enhanced Chemiluminescence Reagent (product of Amersham Pharmacia Biotech) and, then, exposed in intimate contact with X-ray film to detect a black exposure 10 band.

As a result, the probe was found to have firmly hybridized with a ca. 10 kbp EcoRI restriction fragment.

(Example 4)

15 The chromosome DNA of Saitoella complicata IFO 10748 was digested with the restriction enzyme EcoRI and electrophoresed through 0.8% agarose and a ca. 10 kbp fragment of the DNA was excised from the gel and purified to prepare a DNA fragment for cloning. Using λ -DASHII Phage Kit (product of Stratagene), the 20 above DNA fragment was inserted into the EcoRI site of its phage and the packaging was made using In Vitro Packaging Kit (Amersham). Escherichia coli XL1-Blue MRA (P2) was infected and layered on NZY plate medium (5 g/L NaCl, 2 g/L MgSO₄·7H₂O, 5 g/L yeast extract, 10 g/L NZ amine, 18 g/L agar (pH 7.5)) 25 together with NZY soft agar (the agar only of NZY plate medium, 8 g/L) for use as a plaque. This was transferred to HYBOND N + filter (product of Amersham), denatured with alkali (0.5 M NaOH, 1.5 M NaCl), neutralized (0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl), dried, and heated at 80 °C for 2 hours.

30 Using 9 filters heated as above, the prehybridization and the hybridization using the chemiluminescence-labeled probe were carried out as in Example 3 and the filters were rinsed. Each filter was then dried and exposed in intimate contact with X-ray film and the phage plaque corresponding to the black 35 exposure spot was separated. The phage of the separated plaque

was used to infect *E. coli* in the same manner as above and transferred to the filter and the hybridization was carried out again for confirmation. As a result, 6 phage clones could be selected.

5 Using a suspension of the phage, PCR was carried out using said Sa-1S and Sa-2AS primers, and as a result, a 145-bp DNA fragment could be detected in 6 clones. Therefore, the phage DNA was prepared from the recombinant λ -DASHII phage particles according to Laboratory Manual for Genetic Engineering (Masami 10 Muramatsu, Maruzen, 1990). For subcloning, the phage DNA thus prepared was digested with the restriction enzymes SalI and SacI and electrophoresed through 0.8% agarose gel. This gel was denatured with alkali (0.5 M NaOH, 1.5 M NaCl) and neutralized (0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl). Then, a HYBOND N + filter 15 (Amersham) was placed on the gel and subjected to Southern transfer using 20 \times SSC overnight. This filter was dried and heated at 80 °C for 2 hours, after which the Southern hybridization and detection were carried out using ECL Direct Nucleic Acid Labeling/Detection System (Amersham Pharmacia 20 Biotech). Thus, using Gold Hybridizatioon Solution (Amersham Pharmacia Biotech), the prehybridization was carried out at 42 °C for 1 hour.

The chemiluminescence-labeled probe was heated at 95 °C for 5 minutes, quenched on ice, and added to the 25 prehybridization solution used for filter prehybridized and the hybridization was carried out at 42 °C for 22 hours. This filter was washed with 0.5 \times SSC containing 6 M urea and 0.4% SDS at 42 °C twice for 20 minutes each and, then, with 2 \times SSC at room temperature twice for 5 minutes each. The filter was immersed 30 in Enhanced Chemiluminescence Reagent (Amersham Pharmacia Biotech) and exposed in intimate contact with X-ray film to detect a black exposure band.

As a result, the probe was found to have intimately hybridized with a ca. 4.5 kb fragment as obtained by digestion 35 with the restriction enzyme SalI and a ca. 3.5 kb fragment as

obtained by digestion with SacI .

The phage DNA was digested with the restriction enzymes SalI and SacI and electrophoresed through 0.8% agarose gel. The restriction fragment corresponding to the position and size of the black exposure band was excised from the gel and purified using a DNA extraction kit (Sephaglas™ Brand Prep Kit; Amersham Pharmacia Biotech). Then, using a DNA sequencer (Model 377, Perkin-Elmer Corp.) and a DNA sequence kit (Perkin-Elmer Corp., ABI PRISM™ BigDye™ Terminator Cycle Sequence Ready Reaction Kit with AmptiTaq™ DNA polymerase, FS), the sequencing was carried out in accordance with the manufacturer's protocol.

As a result, it was found that the SalI site and SacI site are located at positions 1124 and 1241, respectively, of SEQ ID NO:1 under SEQUENCE LISTING and that neither fragment contained the C-terminal. So, for SalI, which is the upstream one of the two restriction enzymes, in the decaprenyl diphosphate synthase gene, the remaining fragments were examined. As a result, a 3 kbp fragment was found to contain a sequence including a terminal region of the SacI fragment up to the termination codon. By analyzing these 3 restriction fragments, the full-length sequence of the decaprenyl diphosphate synthase gene could be elucidated. Of the three DNA fragments, the ca 1.6 kbp fragment was sequenced. The result is shown as SEQ ID NO:1 under SEQUENCE LISTING. Moreover, the amino acid sequence deduced from the above DNA sequence is shown as SEQ ID NO:2.

Comparison of the DNA sequence thus obtained with that of the decaprenyl diphosphate synthase gene of Saccharomyces cerevisiae as described in Journal of Biological Chemistry, 265, 30 13157-13164 (1990) revealed about 48% homology on the amino acid level as analyzed using Hitachi Soft Engineering's DNASIS software. Comparison with the decaprenyl diphosphate synthase derived from Schizosaccharomyces pombe as described in Japanese Kokai Publication Hei-9-173076 by means of DNASIS revealed 49% 35 homology on the amino acid level.

(Example 5)

In order to selectively excise the gene region coding for decaprenyl diphosphate synthase from the prepared phage DNA, 5 PCR was carried out using synthetic DNA primers Sa-N1 (which has the sequence of 5'-AACATATGGCCTCACCAAGCACTGC GG-3') and Sa-C (which has the sequence of 5'-AAGAATTCCATCTTGACCTAGTCAACAC-3') in otherwise the same manner as in Example 3. After digestion with the restriction 10 enzymes NdeI and EcoRI, the fragment was inserted into the expression vector pUCNT (disclosed in WO 94/03613) to construct the decaprenyl diphosphate synthase gene expression vector pNTSal. The restriction map of the expression vector pNTSal thus obtained is shown in Fig. 1. It is to be noted that DPS 15 represents the coding region of the decaprenyl diphosphate synthase gene.

(Example 6)

The decaprenyl diphosphate synthase gene expression 20 vector pNTSal constructed as above was introduced into Escherichia coli DH5 α . The microorganism was shake-cultured in 10 mL of LB broth at 37 °C overnight and the cells were harvested by centrifugation (3000 rpm, 20 min.).

The cells were suspended in 1 mL of 3% aqueous solution 25 of sulfuric acid and heat-treated at 120 °C for 30 minutes. Then, 2 mL of 14% aqueous solution of sodium hydroxide was added and the mixture was further heat-treated at 120 °C for 15 minutes. The lysate obtained was extracted with 3 mL of hexane-isopropyl alcohol (10:2), and after centrifugation, 1.5 mL of the organic 30 layer was separated and evaporated to dryness under reduced pressure. The residue was dissolved in 200 μ L of ethanol and a 20 μ L portion of the solution was subjected to HPLC analysis (LC-10A, Shimadzu Corporation). Fractionation was carried out using a reversed-phase column (YMC-pack ODS-A, 250 \times 4.6 mm, 35 S-5 μ m, 120 A) and, as the mobile phase, ethanol-methanol (2:1)

and the coenzyme Q₁₀ produced was detected from the absorbance at the wavelength of 275 nm. The result is shown in Fig. 2. As can be seen from Fig. 2, it was found that when the decaprenyl diphosphate synthase gene is introduced into a host and allowed 5 to be expressed, the resulting recombinant Escherichia coli produces coenzyme Q₁₀ which E. coli in general inherently does not produce.

The recombinant E. coli DH5 α (pNTSal) obtained as above has been deposited with National Institute of Bioscience and 10 Human-Technology (Higashi 1-1-3, Tsukuba-shi, Ibaraki, Japan) as of Heisei 11, August 17 (accession number FERM BP-6844).

(Example 7)

The octaprenyl diphosphate synthase gene-knockout 15 Escherichia coli K0229 constructed by Kawamukai et al. is known to retain the gene supported on the spectinomycin-resistant plasmid (pKA3) and die on dropout of said plasmid (Journal of Bacteriology, 179, 3058-3060 (1997)). The pNTSal was introduced into the above knockout strain, culturing the 20 microorganism in 10 mL of ampicillin-containing LB broth by shake culture at 37 °C overnight, subculturing 1% of the culture in 10 mL of fresh ampicillin-containing LB broth and culturing the microorganism further by shake-culture at 37 °C overnight, and after 9 cycles of the above cultural procedure, selecting 25 the strain growing on ampicillin-containing LB plate medium but not growing on pectinomycin-containing LB plate medium.

(Example 8)

The pNTSal-transfected strain constructed in Example 7 30 was shake-cultured in 10 mL of LB broth at 37 °C overnight and the cells were harvested by centrifugation (3000 rpm, 20 min.).

The cells were suspended in 1 mL of 3% aqueous solution of sulfuric acid and heat-treated at 120 °C for 30 min. Then, 2 mL of 14% aqueous solution of sodium hydroxide was added and 35 the mixture was further heat-treated at 120 °C for 15 minutes.

The lysate cells were extracted with 3 mL of hexane-isopropyl alcohol (10:2) and centrifuged to recover 1.5 mL of the organic layer and the solvent was evaporated to dryness under reduced pressure. The residue was dissolved in 200 μ L of ethanol and 5 a 20 μ L portion of the solution was subjected to HPLC analysis (LC-10A, Shimadzu Corporation). Fractionation was carried out by using a reversed-phase column (YMC-pack ODS-A, 250 \times 4.6 mm, S-5 μ m, 120 A) and, as the mobile phase, ethanol-methanol (2:1) and the product coenzyme Q₁₀ was detected from the absorbance 10 at the wavelength of 275 nm. The result is shown in Fig. 3. It is clear from Fig. 3 that as the decaprenyl diphosphate synthase gene was introduced and allowed to be expressed, the host Escherichia coli was enabled to produce coenzyme Q₁₀ which it inherently does not produce and enabled to be transformed 15 so as to yield coenzyme Q₁₀ in an increased amount more than that of the coenzyme Q₈ producer E. coli strain.

INDUSTRIAL APPLICABILITY

The gene coding for the key enzyme associated with the 20 biosynthesis of coenzyme Q₁₀, namely decaprenyl diphosphate synthase, was isolated from fungi of the genus Saitoella and its nucleotide sequence was elucidated. Furthermore, the gene was successfully introduced and expressed in Escherichia coli. By utilizing the technology of the invention, coenzyme Q₁₀ in 25 use as a pharmaceutical can be produced with improved efficiency.

CLAIMS

1. A DNA of the following (a), (b) or (c):

- (a) a DNA having the nucleotide sequence shown under SEQ ID
5 NO:1
- (b) a DNA having a nucleotide sequence derived from the
nucleotide sequence of SEQ ID NO:1 by the deletion, addition,
insertion and/or substitution of one or a plurality of
nucleotides
- 10 and coding for a protein having decaprenyl diphosphate
synthase activity
- (c) a DNA which hybridizes with the DNA having the nucleotide
sequence of SEQ ID NO:1 under a stringent condition
and codes for a protein having decaprenyl diphosphate
15 synthase activity.

2. A protein of the following (d) or (e):

- (d) a protein having the amino acid sequence shown under SEQ
ID NO:2
- 20 (e) a protein having an amino acid sequence derived from the
amino acid sequence of SEQ ID NO:2 by the deletion, addition,
insertion and/or substitution of one or a plurality of amino
acids and having decaprenyl diphosphate synthase activity.

25 3. A DNA coding for the protein according to Claim 2.

4. An expression vector constructed by cloning the DNA
according to Claim 1 or 3 in an expression vector.

30 5. The expression vector according to Claim 4
wherein the expression vector is pUCNT.

6. The expression vector according to Claim 5
wherein the expression vector is pNTSal.

7. A transformant as obtainable by transforming a host microorganism with the DNA according to Claim 1 or 3.

8. A transformant as obtainable by transforming a host 5 microorganism using the expression vector according to Claim 4, 5 or 6.

9. The transformant according to Claim 7 or 8 wherein the host microorganism is Escherichia coli.
10

10. The transformant according to Claim 9 wherein the Escherichia coli is Escherichia coli DH5_α.

15 11. The transformant according to Claim 10 which is E. coli DH5_α (pNTSal) (FERM BP-6844).

12. A process for producing a coenzyme Q₁₀ which comprises culturing the transformant according to 20 Claim 7, 8, 9, 10 or 11 in a culture broth and harvesting the coenzyme Q₁₀ produced and accumulated in the resulting culture.

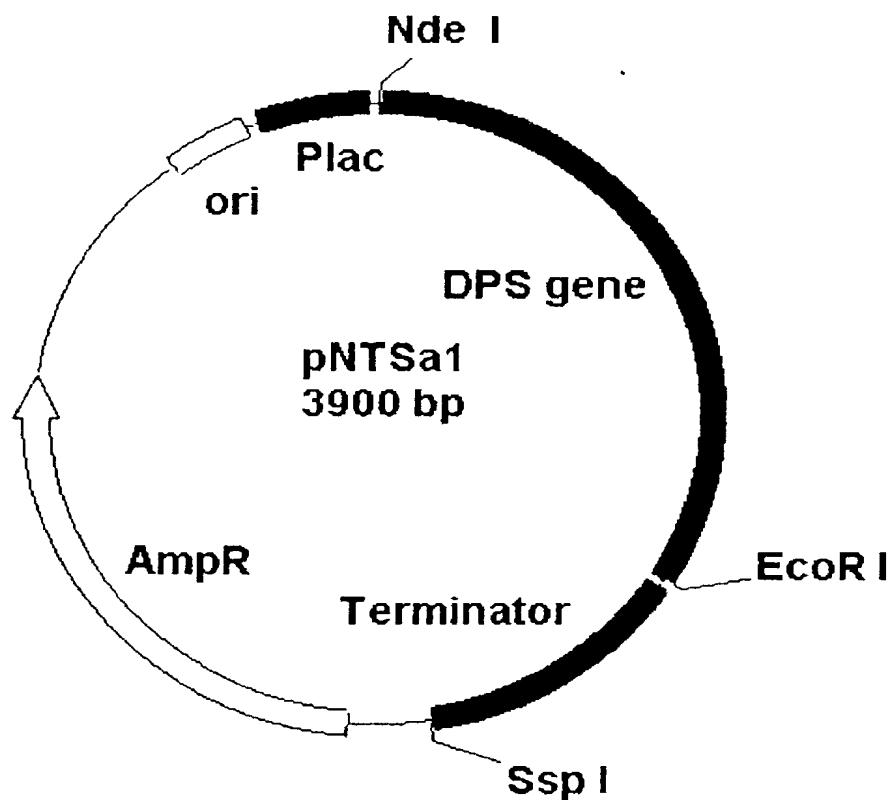
ABSTRACT

The present invention has for its object to isolate a gene coding for the enzyme synthesizing the coenzyme Q₁₀ side chain 5 from a fungal strain of the genus Saitoella and exploit it to advantage for the efficient microbial production of coenzyme Q₁₀.

- The present invention provides;
- a DNA having the nucleotide sequence shown under SEQ ID 10 NO:1
- a DNA having a nucleotide sequence derived from the nucleotide sequence of SEQ ID NO:1 by the deletion, addition, insertion and/or substitution of one or a plurality of nucleotides
- 15 and coding for a protein having decaprenyl diphosphate synthase activity
- a DNA which hybridizes with the DNA having the nucleotide sequence of SEQ ID NO:1 under a stringent condition
- 20 and codes for a protein having decaprenyl diphosphate synthase activity.

1/3

Fig. 1

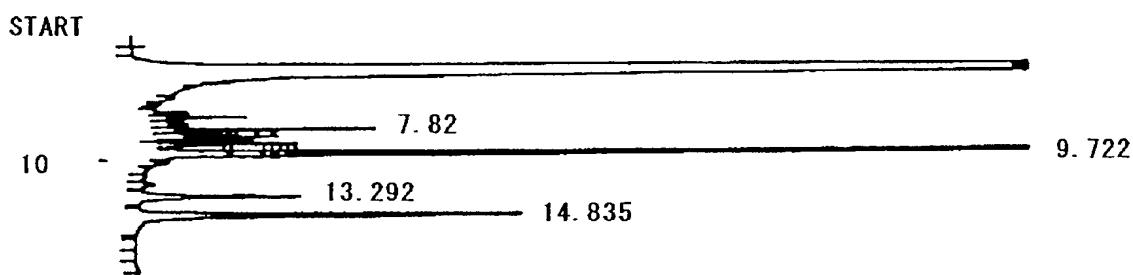


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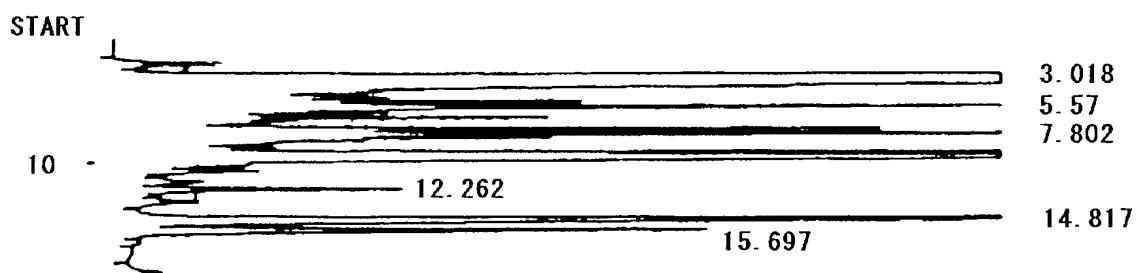
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Fig. 2

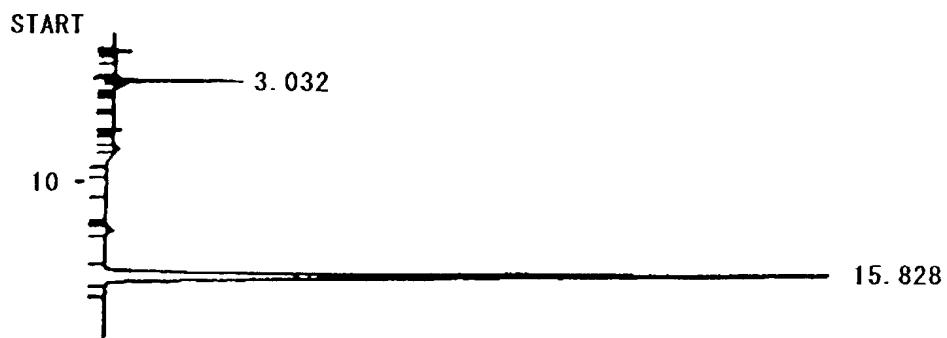
E. Coli DH5 α



E. Coli DH5 α / pNTSa1



CoQ₁₀ Standard

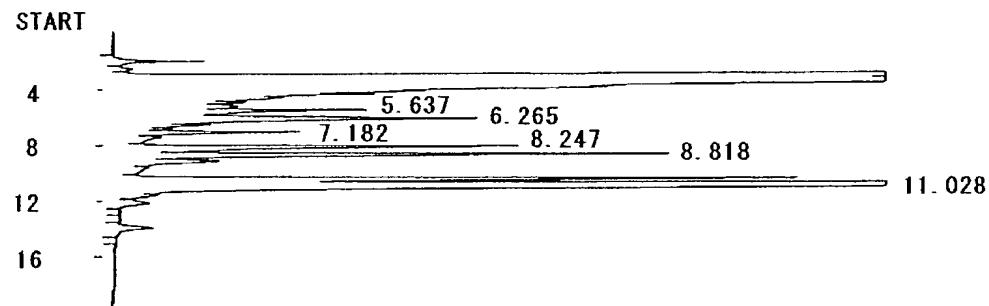


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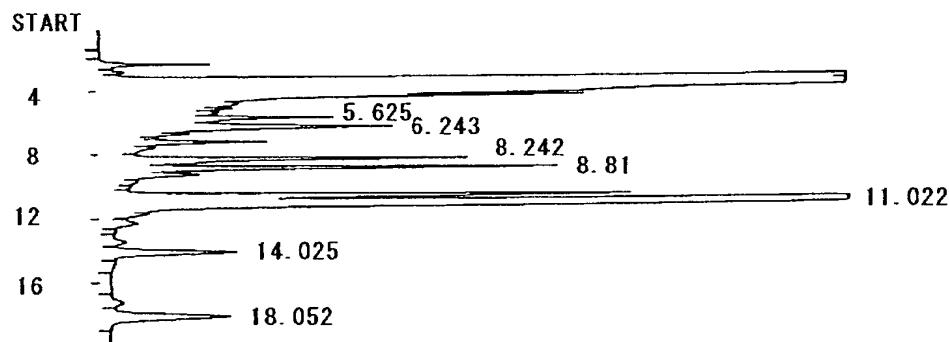
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Fig. 3

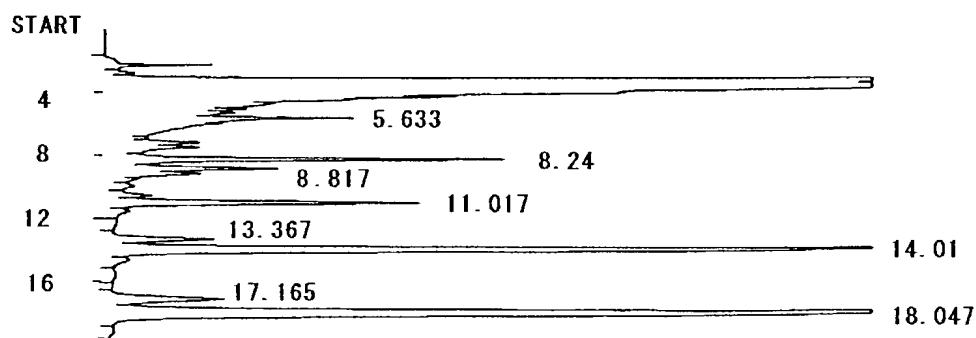
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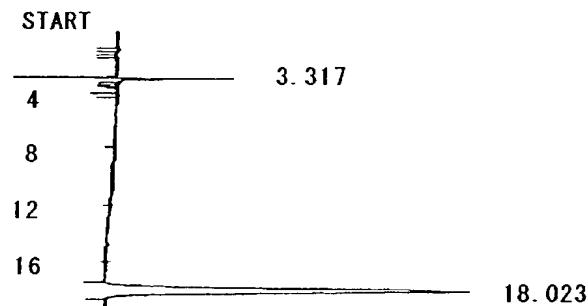
E. Coli K0229 / pKA3 + pNTSa1



E. Coli K0229 / pNTSa1



CoQ₁₀ Standard



DECLARATION FOR PATENT APPLICATION

1581/00265

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR PRODUCING COENZYME Q10

the specification of which: (check one)

is attached hereto. was filed on August 24, 2000, as United States Patent Application Serial No. or PCT International Application Number PCT/JP00/05659, and was amended on 19 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with 37 CFR § 1.56(a).

Prior Foreign Application(s): I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate listed below, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

Priority Claimed			
11/237561 (Application No.)	Japan (Country)	24/August/1999 (Day/Month/Year Filed)	[XX] YES [] YES
<hr/>	<hr/>	<hr/>	<hr/>
<hr/>	<hr/>	<hr/>	<hr/>

I hereby claim the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below:

Application No.	Filing Date
<hr/>	<hr/>

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s) listed below or 34 U.S.C. § 365(c) of any PCT International Application designating the United States of America listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application or PCT application in the manner provided by 35 U.S.C. § 112, first paragraph, I acknowledge the duty to disclose material information as defined in 37 CFR § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(U.S. or PCT Application Serial No.)	(U.S. or PCT Filing Date)	(Status - patented, pending, abandoned)
--------------------------------------	---------------------------	---

21 I hereby appoint the following registered practitioners: Rudolf E. Hutz, Reg. No. 22,397; John D. Fairchild, Reg. No. 19,756; Harold Pezzner, Reg. No. 22,112; Richard M. Beck, Reg. No. 22,580; Paul E. Crawford, Reg. No. 24,397; Burton A. Amernick, Reg. No. 24,852; Morris Liss, Reg. No. 24,510; George R. Pettit, Reg. No. 27,369; Patricia Smink Rogowski, Reg. No. 33,791; Robert G. McMorrow, Jr., Reg. No. 30,962; Ashley I. Pezzner, Reg. No. 35,646; William E. McShane, Reg. No. 32,707; Mary W. Bourke, Reg. No. 30,982; Gerard M. O'Rourke, Reg. No. 39,794; James M. Olsen, Reg. No. 40,408; Francis DiGiovanni, Reg. No. 37,310; Eric J. Evain, Reg. No. 42,517; William E. Curry, Reg. No. 43,572; Daniel C. Mulveny, Reg. No. 45,897; John A. Evans, (Agent) 44,100; and Elliot C. Mendelson (Agent), Reg. No. 42,878, with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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2-00

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Sequence listing

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15 <211> 1653

<212> DNA

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Ile Arg Pro Leu Met Val Leu Leu Met Ala Gln Ala Thr Glu Val Ala

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Gly Leu Ala Pro Pro Glu Val Leu Asn Asp Lys Asn Pro Asp Met Met

10 145 150 155

aac atg agg tca gga cca tta acg aag gac ggc gag atc gag gga cag 649

Asn Met Arg Ser Gly Pro Leu Thr Lys Asp Gly Glu Ile Glu Gly Gln

160 165 170

15

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Thr Ser Asn Ile Leu Ala Ser Gln Arg Arg Leu Ala Glu Ile Thr Glu

175 180 185

20 atg atc cat gca gca tca ctc ctc cac gac gac gtt atc gac gct tcc 745

Met Ile His Ala Ala Ser Leu Leu His Asp Asp Val Ile Asp Ala Ser

190 195 200

gag acc aga cga aac gca cca tcc gga aac cag gca ttc gga aac aag 793

25 Glu Thr Arg Arg Asn Ala Pro Ser Gly Asn Gln Ala Phe Gly Asn Lys

205 210 215 220

atg gcg att ttg gct ggt gat ttc ttg ttg gga cgg gcg tct gtt gca 841
Met Ala Ile Leu Ala Gly Asp Phe Leu Leu Gly Arg Ala Ser Val Ala

5

ttg gcg agg ttg cgc aat ccg gag gtg att gag ctt ttg gct act gtt 889
Leu Ala Arg Leu Arg Asn Pro Glu Val Ile Glu Leu Leu Ala Thr Val

240 245 250

10 att gca aac ttg gtt gag gga gag ttc atg cag ttg aaa aat act gtt 937
Ile Ala Asn Leu Val Glu Gly Glu Phe Met Gln Leu Lys Asn Thr Val

255 260 265

gat gat gcg att gag gct acg gcg acg cag gaa acg ttc gat tac tat 985
15 Asp Asp Ala Ile Glu Ala Thr Ala Thr Gln Glu Thr Phe Asp Tyr Tyr

270 275 280

ttg cag aag act tac ttg aag act gcg tcc ttg att gcc aag tcg tgc 1033
 Leu Gln Lys Thr Tyr Leu Lys Thr Ala Ser Leu Ile Ala Lys Ser Cys
 20 285 290 295 300

aga gca agt gcg ctt ctg ggt ggt gct acg cct gag gtt gct gat gct 1081
Arg Ala Ser Ala Leu Leu Gly Gly Ala Thr Pro Glu Val Ala Asp Ala

305 310 315

25

gct tat gct tac gga agg aac ctt ggt ttg gca ttc caq atc gtc gac 1129

Ala Tyr Ala Tyr Gly Arg Asn Leu Gly Leu Ala Phe Gln Ile Val Asp

320 325 330

gac atg ctc gac tac acc gtc tcc gct acc gac ctc ggt aag ccc gcc 1177

5 Asp Met Leu Asp Tyr Thr Val Ser Ala Thr Asp Leu Gly Lys Pro Ala
335 340 345

ggt gca gac ctc cag ctc ggt ctc gcc acc gcg ccc gcc ctc ttc gca 1225

Gly Ala Asp Leu Gln Leu Gly Leu Ala Thr Ala Pro Ala Leu Phe Ala

10 350 355 360

tgg aag cac cac gcc qaq ctc qgt ccc atg atc aag cgc aag ttc tct 1273

Trp Lys His His Ala Glu Leu Gly Pro Met Ile Lys Arg Lys Phe Ser

15

gac cca gga gac gtc gaa cgt gca cgc gaa ttg gtc gaa aaa aat gat 1321

Asp Pro Gly Asp Val Glu Arg Ala Arg Glu Leu Val Glu Lys Ser Asp

20 gga ttg gag aag acc aga ggc ttg gca gag gag tat ggc gca aag gca 1368

Gly Leu Glu Lys Thr Arg Ala Leu Ala Glu Glu Tyr Ala Cln Lys Ala

400 405 410

ttg gat gca att cgg acg ttc cca gag aat cca gca ccc aag gat ttg 1417

25 Leu Asp Ala Ile Arg Thr Phe Pro Glu Ser Pro Ala Asp Ile Asp Ile

415 420 425

gag cag ttg acg gac aag gtg ttg act agg tca aga taggaattcgagct 1467
 Glu Gln Leu Thr Asp Lys Val Leu Thr Arg Ser Arg
 430 435 440

5
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 atgagagaag attttcagcc tgatacagat taaatcagaa cgcagaagcg gtctgataaa 1587

10 acagaatttg cctggcggca gtagcgcggt ggtcccacct gacccatgc cgaactcaga 1647
 agtgaa 1653

15 <210> 2
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 <212> PRT
 <213> Saioella complicata

20 <400> 2
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 Ile Ala Ser Leu Arg Ser Val Thr Leu Arg Thr Ala Ser Ala Pro
 20 25 30

25 Ser Leu Arg Leu Arg Cys Thr Pro Thr Ser Arg Pro Ser Ser Ser
 35 40 45

Trp	Ala	Ala	Ala	Val	Ser	Ser	Ala	Ser	Arg	Leu	Val	Glu	Pro	Asp	
				50					55				60		
Pro	Asn	Gln	Pro	Leu	Ile	Asn	Pro	Leu	Asn	Leu	Val	Gly	Pro	Glu	
				65					70				75		
5	Met	Ser	Asn	Leu	Thr	Ser	Asn	Ile	Arg	Ser	Leu	Leu	Gly	Ser	Gly
				80					85				90		
His	Pro	Ser	Leu	Asp	Thr	Val	Ala	Lys	Tyr	Tyr	Val	Gln	Ser	Glu	
				95					100				105		
Gly	Lys	His	Ile	Arg	Pro	Leu	Met	Val	Leu	Leu	Met	Ala	Gln	Ala	
10				110					115				120		
Thr	Glu	Val	Ala	Pro	Lys	Val	Gln	Gly	Trp	Glu	Lys	Val	Val	Glu	
				125					130				135		
Val	Pro	Val	Asn	Glu	Gly	Leu	Ala	Pro	Pro	Glu	Val	Leu	Asn	Asp	
				140					145				150		
15	Lys	Asn	Pro	Asp	Met	Met	Asn	Met	Arg	Ser	Gly	Pro	Leu	Thr	Lys
				155					160				165		
Asp	Gly	Glu	Ile	Glu	Gly	Gln	Thr	Ser	Asn	Ile	Leu	Ala	Ser	Gln	
				170					175				180		
Arg	Arg	Leu	Ala	Glu	Ile	Thr	Glu	Met	Ile	His	Ala	Ala	Ser	Leu	
20				185					190				195		
Leu	His	Asp	Asp	Val	Ile	Asp	Ala	Ser	Glu	Thr	Arg	Arg	Asn	Ala	
				200					205				210		
Pro	Ser	Gly	Asn	Gln	Ala	Phe	Gly	Asn	Lys	Met	Ala	Ile	Leu	Ala	
				215					220				225		
25	Gly	Asp	Phe	Leu	Leu	Gly	Arg	Ala	Ser	Val	Ala	Leu	Ala	Arg	Leu
				230					235				240		

Arg Asn Pro Glu Val Ile Glu Leu Leu Ala Thr Val Ile Ala Asn		
245	250	255
Leu Val Glu Gly Glu Phe Met Gln Leu Lys Asn Thr Val Asp Asp		
260	265	270
5 Ala Ile Glu Ala Thr Ala Thr Gln Glu Thr Phe Asp Tyr Tyr Leu		
275	280	285
Gln Lys Thr Tyr Leu Lys Thr Ala Ser Leu Ile Ala Lys Ser Cys		
290	295	300
Arg Ala Ser Ala Leu Leu Gly Gly Ala Thr Pro Glu Val Ala Asp		
10 305	310	315
Ala Ala Tyr Ala Tyr Gly Arg Asn Leu Gly Leu Ala Phe Gln Ile		
320	325	330
Val Asp Asp Met Leu Asp Tyr Thr Val Ser Ala Thr Asp Leu Gly		
335	340	345
15 Lys Pro Ala Gly Ala Asp Leu Gln Leu Gly Leu Ala Thr Ala Pro		
350	355	360
Ala Leu Phe Ala Trp Lys His His Ala Glu Leu Gly Pro Met Ile		
365	370	375
Lys Arg Lys Phe Ser Asp Pro Gly Asp Val Glu Arg Ala Arg Glu		
20 380	385	390
Leu Val Glu Lys Ser Asp Gly Leu Glu Lys Thr Arg Ala Leu Ala		
395	400	405
Glu Glu Tyr Ala Gln Lys Ala Leu Asp Ala Ile Arg Thr Phe Pro		
410	415	420
25 Glu Ser Pro Ala Arg Lys Ala Leu Glu Gln Leu Thr Asp Lys Val		
425	430	435

Leu Thr Arg Ser Arg

440